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Heme oxygenase-1 protects against radiocontrast-induced acute kidney injury by regulating anti-apoptotic proteins

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Radiocontrast agents are thought to induce acute kidney injury in part through increased production of reactive oxygen species and increased cellular apoptosis. In this study we determined whether heme oxygenase-1 could prevent or reduce radiocontrast-induced acute kidney injury and, if so, what were the mechanisms by which this can occur. Sodium iothalamate was administered to uninephrectomized, salt-depleted male Sabra rats to initiate acute kidney injury. Heme oxygenase-1 was induced with cobalt protoporphyrin or inhibited with stannous mesoporphyrin. Inhibition of heme oxygenase exacerbated kidney injury as measured by an increase in plasma creatinine and in superoxide production. Heme oxygenase-1 induction prevented the increase in plasma creatinine and in superoxide in both the cortex and medulla compared to untreated rats with acute kidney injury. This protective effect of heme oxygenase-1 was associated with increased anti-apoptotic proteins Bcl-2 and Bcl-xl and a decrease of pro-apoptotic caspase-3 and caspase-9 along with increased expression of inactive BAX. Our study suggests that increased levels of heme oxygenase-1 are protective against acute kidney injury due to radiocontrast exposure.

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Heme oxygenase (HO), as the key enzyme in heme degradation, not only controls the cellular levels of heme available for the synthesis of heme proteins but is also responsible for the generation of bilirubin, an antioxidant, and the active gas, carbon monoxide (CO).[1–3] Two HO isoforms (HO-1 and HO-2), the products of two distinct genes, have been shown to be important in the catabolism of heme in mammals. HO-2, which is constitutively expressed, is localized primarily in the brain, testis, and the vascular endothelium.[1] HO-1, the inducible form, has been shown to protect the kidney in several models of acute kidney injury (AKI), such as ischemia- and glycerol-induced AKI, nephrotoxic serum nephritis, cisplatin nephrotoxicity, and rapamycin-induced AKI.[4–8] In the glycerol model of AKI, an increased release of heme was shown to cause renal toxicity, whereas induction of HO-1 preserved renal function and reduced inflammatory molecules.[9]

The beneficial effects of HO-1 induction in AKI are thought to occur via several mechanisms. Increased HO-1 activity results in degradation of the heme moiety, which is a pro-oxidant and potentially toxic to several cellular targets, including lipid bilayers, mitochondria, cytoskeleton, and components of the nucleus.[10–12] In addition to detoxifying heme, elevated HO-1 activity results in the increased generation of bilirubin, an antioxidant capable of scavenging peroxy radicals and inhibiting lipid peroxidation.[2] Finally, HO-1 generates CO gas as a byproduct of the breakdown of heme. Several studies have demonstrated the protective role of CO itself in limiting renal damage in ischemia-induced AKI.[13–15]

A potential mechanism of AKI is thought to be mediated by the increased production of reactive oxygen species and increased tubular cell apoptosis.[16,17] Recent studies by Yano *et al.*[18] have reported that the radiocontrast agent, ioversol, inhibits the cyclic adenosine mono phosphate-stimulated phosphorylation of Akt, a kinase that when activated can enhance Bcl-2 expression, and that stimulation of the cyclic adenosine mono phosphate pathway to increase Akt phosphorylation can prevent ioversol-induced apoptosis in cultured renal tubular cells. Akt is known to be activated in

response to increased HO-1 or CO generation and may play an important role in preventing apoptosis.[19–22] The effects of HO-1 gene expression and activity on the Bcl-2 family of pro- and anti-apoptotic proteins, which may be involved in the restoration of renal function in AKI, remain to be determined.

The goals of this study were to determine whether HO-1 induction is protective of renal function in radiocontrast-induced AKI and if HO-1 induction inhibits superoxide (O_2^-) production and the apoptotic signaling pathways activated by AKI. Our results indicate a key role for HO-1 induction in the prevention of radiocontrast-induced AKI through mechanisms that involve inhibition of reactive oxygen species generation as well as suppression of pro-apoptotic and stimulation of anti-apoptotic proteins.

RESULTS

Effect of AKI on HO-1 and HO-2

The induction of AKI led to an increase in HO-1 protein in the cortex, but not in the medulla, while no significant differences in HO-2 protein expression were observed (Figure 1a). Treatment with cobalt protoporphyrin (CoPP) significantly increased HO-1 protein levels in the cortex and in the medulla, but had no effect on the levels of HO-2 protein (Figure 1b). Stannous mesoporphyrin (SnMP) also increased the levels of HO-1 protein.

Renal HO activity and heme measurements

To further define the effect of radiocontrast-induced AKI on renal HO-1, we measured HO activity in control and AKI rats before and after administration of CoPP or SnMP. HO activity was 0.71 ± 0.032 nmol bilirubin/mg/h in controls compared to 0.835 ± 0.057 nmol bilirubin/mg/h in AKI rats (Figure 2a). Treatment with CoPP increased HO activity to 2.12 ± 0.12 nmol bilirubin/mg/h, which was a 2.5-fold increase in HO activity compared to nontreated AKI controls ($P < 0.05$). Weekly administration of SnMP decreased HO activity by 50% (to 0.34 ± 0.026 nmol bilirubin/mg/h) despite the fact that it increased HO-1 protein levels (Figure 2a).

As seen in Figure 2b, renal heme content was significantly increased by kidney injury (0.61 ± 0.058 in control rats vs 0.95 ± 0.021 nmol/mg protein in AKI rats). Treatment with

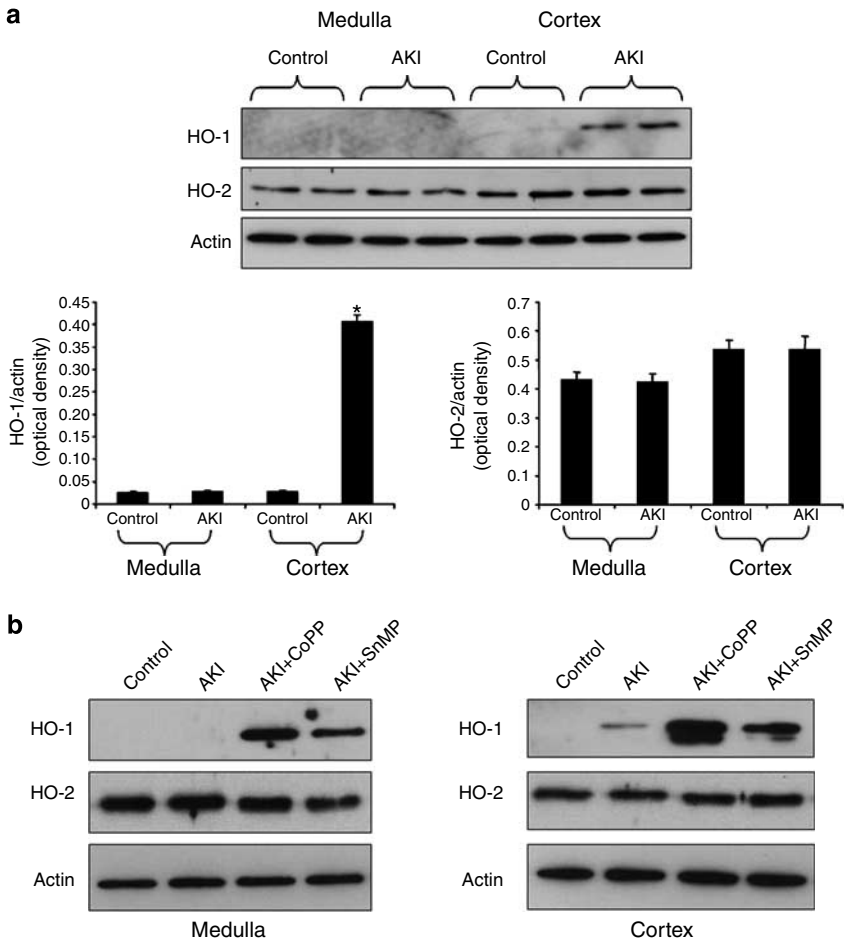

Figure 1 | Effect of AKI and CoPP on HO-1 and HO-2 expression. (**a**) Western blot and densitometry analysis of HO-1, HO-2, and actin in the renal medulla and renal cortex from control and AKI rats. Results are expressed as mean ± s.e., $n = 4$, *$P < 0.05$ vs control. (**b**) Western blot of HO-1, HO-2, and actin in the renal medulla and renal cortex from control, AKI, AKI + CoPP-, and AKI + SnMP-treated rats (representative of three experiments). 254 × 190 mm (300 × 300 d.p.i.).

CoPP lowered renal heme content to 0.28 ± 0.048 nmol/mg protein, while inhibition of HO with SnMP further exacerbated the increase in renal heme content (averaging 1.141 ± 0104 nmol/mg protein) compared to nontreated AKI rats.

Renal levels of eNOS and iNOS protein

AKI led to a dramatic reduction of endothelial nitric oxide synthase (eNOS) protein while, at the same time, increasing the levels of inducible nitric oxide synthase (iNOS) protein in the kidney (Figure 3). Treatment with CoPP restored the levels of eNOS protein to those observed in control rats and was also able to attenuate the increase in iNOS protein levels observed in AKI rats (Figure 3). SnMP treatment increased the levels of iNOS protein in response to AKI, but had no effect on eNOS levels.

Effect of HO-1 expression on O_2^- anion

Compared to control rats, kidney injury significantly increased O_2^- production by about 1.8-fold from 5.85 ± 0.5 to 10.53 ± 0.6 μmol O_2^-/mg protein (Figure 4). Induction of HO-1 with CoPP completely prevented the increase of O_2^- production by AKI, averaging 4.72 ± 0.4 μmol O_2^-/mg protein ($P<0.05$). In contrast, inhibition of HO-1 with SnMP significantly increased O_2^- production to 12.48 ± 0.7 μmol O_2^-/mg protein (Figure 4).

Effect of HO-1 expression on renal function

As seen in Figure 5a, the induction of AKI resulted in an increase in plasma creatinine to 0.55 ± 0.08 mg% compared to 0.42 ± 0.01 mg% in controls ($P<0.05$). CoPP treatment prevented the increase in plasma creatinine (0.32 ± 0.06 mg%; $P<0.05$), while rats treated with SnMP plasma creatinine was similar to untreated AKI, 0.59 ± 0.09 mg%. Glomerular filtration rate, as measured by the clearance of creatinine, was significantly reduced in AKI rats compared to controls, 1.84 ± 0.26 vs 3.98 ± 0.87 ml/min, $P<0.05$ (Figure 5b). Creatinine clearance was significantly higher after the induction of HO-1 with CoPP, averaging 2.86 ± 0.77 ml/min, while treatment with SnMP had no affect on creatinine clearance, averaging 2.11 ± 0.37 ml/min.

Effect of HO-1 induction on pro- and anti-apoptotic protein levels

AKI resulted in a significant increase in caspase-3 and caspase-9 activities in the medulla (Figure 6). Since HO-1 has been shown to modulate the apoptotic pathway,[23] we examined the CoPP- and SnMP-mediated modulation of HO

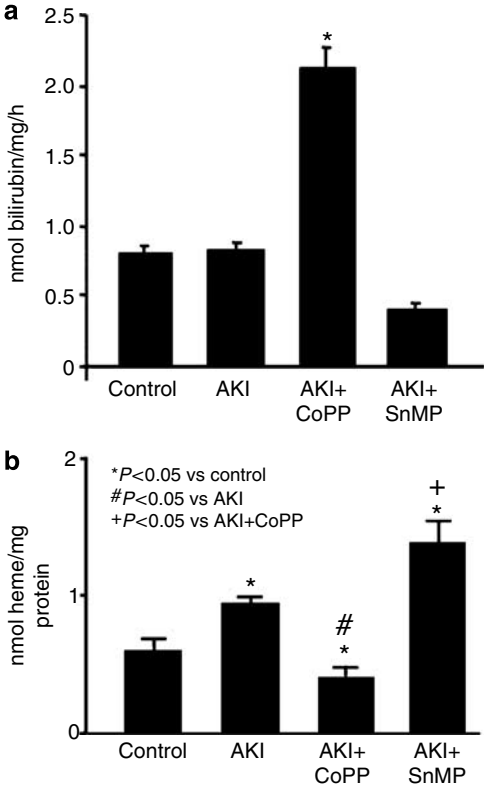

Figure 2 | Effect of AKI and CoPP on HO activity and heme content. (**a**) HO activity in the renal cortex of control, AKI-, AKI + CoPP-, and AKI + SnMP-treated rats. (**b**) Heme content in the renal cortex of control, AKI-, AKI + CoPP-, and AKI + SnMP-treated rats. Results are expressed as mean ± s.e., $n = 6$, $^*P<0.05$ vs control, $^{\#}P<0.05$ vs AKI, $^{\dagger}P<0.05$ vs AKI + CoPP. 254 × 190 mm (300 × 300 d.p.i.).

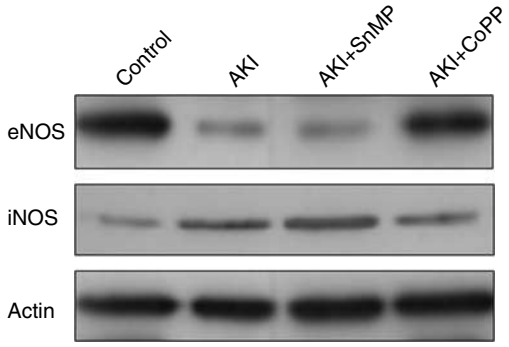

Figure 3 | Western blot of eNOS and iNOS in the kidney of control, AKI-, AKI + CoPP-, and AKI + SnMP-treated rats (representative of three experiments). 254 × 190 mm (300 × 300 d.p.i.).

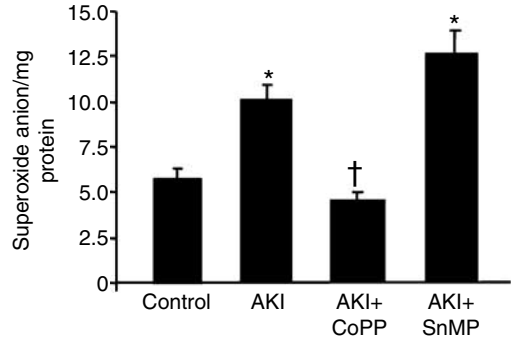

Figure 4 | Superoxide anion (O_2^-) production in the renal cortex of control, AKI-, AKI + CoPP-, and AKI + SnMP-treated rats. Results are expressed as mean ± s.e., $n = 6$, $^*P<0.05$ vs control, $^{\dagger}P<0.05$ vs AKI and AKI + CoPP. 254 × 190 mm (300 × 300 d.p.i.).

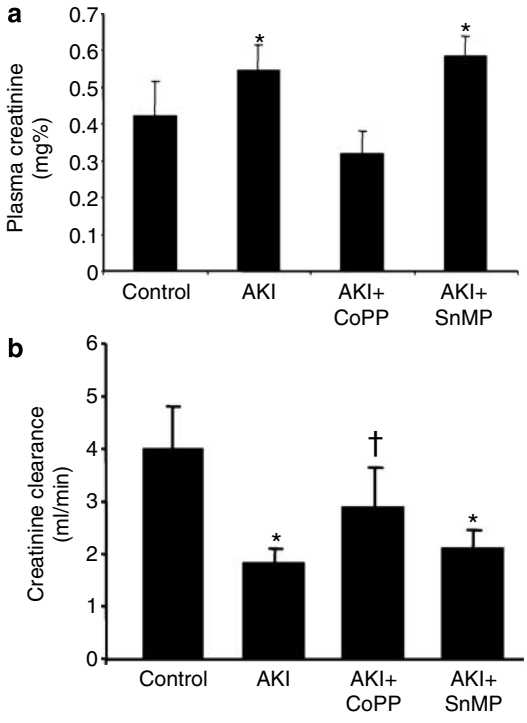

Figure 5 | Effect of AKI and HO-1 induction on plasma creatinine and creatinine clearance. (**a**) Plasma creatinine in control, AKI-, AKI + CoPP- and AKI + SnMP-treated rats. (**b**) Creatinine clearance in control, AKI-, AKI + CoPP-, and AKI + SnMP-treated rats. Results are expressed as mean ± s.e., $n = 6$, $^{*}P < 0.05$ vs control, $^{\dagger}P < 0.05$ vs AKI + SnMP. 254 × 190 mm (300 × 300 d.p.i.).

activity. As seen in Figure 6, the induction of HO-1 by CoPP strongly abrogated the increase in caspase-3 and caspase-9 activity in the medulla, while there was no significant difference in the cortex. SnMP, an inducer of HO-1 protein but inhibitor of CO and bilirubin, did not decrease caspase levels. These results suggest that it is not HO-1 protein, but HO-1-derived CO and bilirubin that regulate caspase activity.

To investigate the possibility that the differential activation of pro-apoptotic and anti-apoptotic proteins may account for the effect seen in HO-1-modulated renal protection, we examined the levels of Bax, Bcl-2, and Bcl-xl proteins in the renal medulla and cortex. The induction of AKI resulted in a significant increase in Bax levels in the medulla. The levels of Bax in the cortex tended to be higher following AKI, but the difference did not reach statistical significance (Figures 7 and 8). The AKI-induced increase in Bax in the medulla was prevented by CoPP treatment (Figures 7 and 8). Notably, treatment with SnMP caused a dramatic augmentation of the AKI-induced increase in Bax levels in both the medulla and cortex (Figures 7 and 8). Treatment with CoPP dramatically increased Bcl-2 expression in the medulla. In the cortex, Bcl-2 expression levels tended to be elevated by CoPP; however, the increase did not reach statistical significance. Importantly, SnMP reduced cortical Bcl-2 levels in AKI rats, even to below those of controls ($P < 0.05$; Figure 8a and b). Compared to controls and nontreated AKI rats, CoPP-treated AKI animals had significantly increased Bcl-xl levels in the medulla (Figure 7) and cortex (Figure 8).

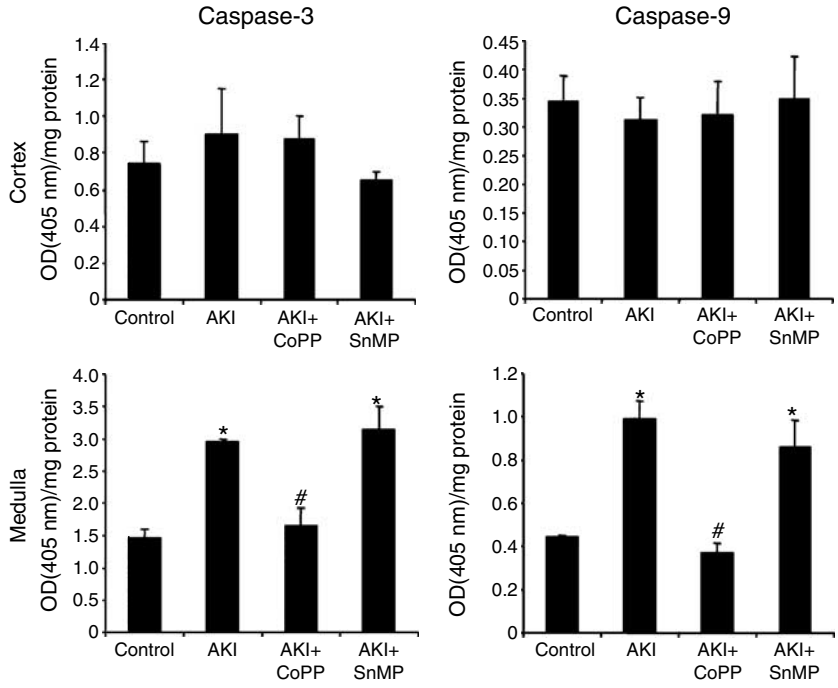

Figure 6 | Caspase-3 and caspase-9 activity in the renal medulla and cortex. Results are expressed as mean ± s.e. ($n = 4$ in each group), $^{*}P < 0.05$ vs control, $^{\#}P < 0.05$ vs AKI. 254 × 190 mm (300 × 300 d.p.i.).

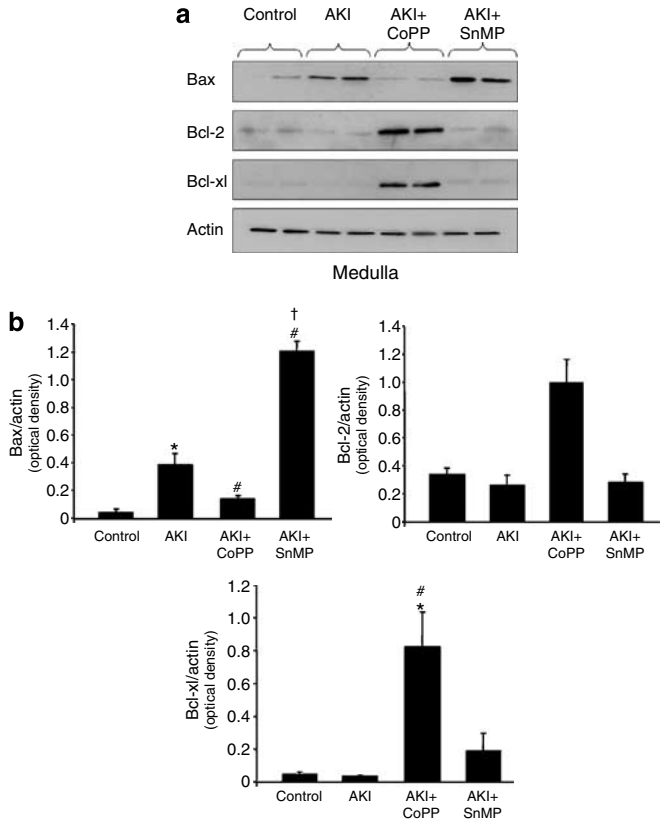

Figure 7 | Effect of AKI and HO-1 expression on anti-apoptotic signaling protein, Bcl-2 and Bcl-xl in the medulla. Western blot (**a**) and densitometry analysis (**b**) of pro- and anti-apoptotic proteins in the renal medulla of control, AKI, AKI + CoPP and AKI + SnMP rats. Results are expressed as mean ± s.e. ($n = 4$ in each group), $^{*}P < 0.05$ vs control, $^{\#}P < 0.05$ vs AKI, $^{\dagger}P < 0.05$ vs AKI + CoPP. 254 × 190 mm (300 × 300 d.p.i.).

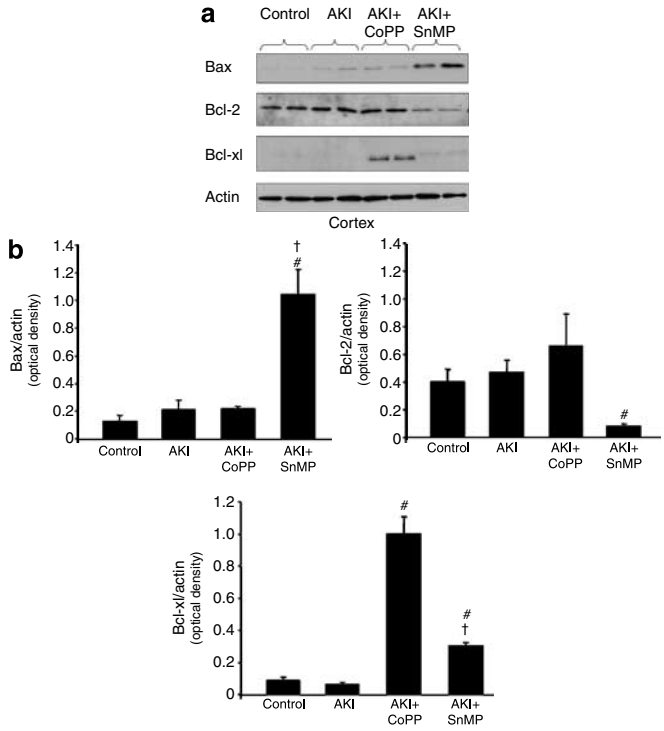

Figure 8 | Effect of AKI and HO-1 expression on anti-apoptotic signaling protein, Bcl-2 and Bcl-xl in the cortex. Western blot (**a**) and densitometry analysis (**b**) of pro- and anti-apoptotic proteins in the renal cortex of control, AKI, AKI + CoPP and AKI + SnMP rats. Results are expressed as mean ± s.e. ($n = 4$ in each group); $^{*}P < 0.05$ vs control, $^{\#}P < 0.05$ vs AKI, $^{\dagger}P < 0.05$ vs AKI + CoPP. 254 × 190 mm (300 × 300 d.p.i.).

DISCUSSION

This study demonstrates that HO-1 plays an important role in renal protection in a radiocontrast model of AKI. Normal renal function was maintained in AKI rats treated with CoPP. CoPP treatment decreased heme levels and O_2^- formation in the kidney, presumably due to the induction of HO-1 and the subsequent increased generation of bilirubin and CO. We have also demonstrated a link between increased levels of anti-apoptotic proteins and the HO-1-mediated prevention of renal dysfunction and inhibition of tubular necrosis as seen by the decrease in caspase activity. CoPP treatment resulted in a robust increase in the anti-apoptotic proteins Bcl-xl and Bcl-2, a decrease in the pro-apoptotic Bax protein, and inhibition of caspase activity. Lastly, the induction of HO-1 by CoPP decreased iNOS levels and increased the levels of eNOS in the kidney in this model of radiocontrast-induced AKI.

Our results clearly demonstrate that induction of HO-1 by CoPP can protect the kidney from radiocontrast-induced AKI. These results are consistent with previous observations that HO-1 expression in renal tissue is cytoprotective in both heme- and non-heme-mediated models of AKI. For example, in a heme-mediated model of AKI associated with rhabdomyolysis, the administration of tin protoporphyrin (SnPP), which is a specific inhibitor of HO, worsened renal damage, while the induction of HO-1 led to a considerable decrease in mortality.[24] In *in vitro* studies with purified HO-1 and HO-2, inhibitors of HO activity such as Sn- and Zn-protoporphyrins were equally inhibitory to HO activity; athought, SnMP was more inhibitory to HO-2-dependent activity than to that of HO-1.[25] In cisplatin-induced nephrotoxicity, inhibition of HO by SnMP significantly worsened both the structural and functional parameters of renal injury,[5] providing direct evidence for the role of HO (most likely HO-1) in protecting the kidney in this model of AKI. Several additional studies have also confirmed the protective role of HO-1 induction in protecting the kidney and have described the effect of HO inhibition as worsening kidney damage following ischemia- and rapamycin-induced AKI.[4,26,27]

Our results demonstrate that induction of HO-1 was associated with maintenance of glomerular filtration rate, as indicated by normal creatinine clearances and plasma creatinine levels in CoPP-treated AKI rats. Previous studies have demonstrated a sharp decline in renal medullary blood flow following administration of radiocontrast agents.[28,29] CO generated from HO is a known vasodilator and has been

linked to the regulation of medullary blood flow in the kidney.[30] It is possible that the induction of HO-1 via CoPP maintains adequate medullary blood flow, thus preventing ischemic injury. In the current study, AKI was also associated with a significant decrease in the level of eNOS protein in the kidney. This decrease was completely prevented by CoPP treatment. It is possible that the decrease in eNOS contributes to the decreased glomerular filtration rate and reduced regional blood flow in the kidney following AKI. Restoration of eNOS by CoPP may be a contributing factor to the improvement of blood flow and the prevention of renal damage in AKI rats. CoPP treatment has been shown to improve vascular function and increase eNOS levels in diabetic rats.[31] Radiocontrast-induced AKI has also been associated with increased production of reactive oxygen species, which may contribute to the elevation in vascular tone and increased apoptosis observed in this pathological condition.[17,32] In the present study, sodium iothalamate-induced AKI led to an increase in O_2^- production in the kidney, which was completely prevented by induction of HO-1 with CoPP. Conversely, inhibition of HO activity with SnMP led to a significant increase in O_2^- production.

HO induction by CoPP also increased the production of another potent antioxidant, bilirubin. Bilirubin has been shown to be protective against oxidative injury in neurons as well as in ischemic–reperfusion injury of the liver.[33,34] Bilirubin has also been shown to inhibit protein kinase C and NAD(P)H oxidase activities.[35–37] Recently, the upregulation of HO-1 gene expression was shown to decrease the availability of the heme-containing gp91 subunit necessary for NADPH oxidase activity and O_2^- generation and to increase bilirubin formation.[38] Furthermore, a decrease in mitochondrial heme as a result of the increase in mitochondrial HO-1 may reduce fatty acid lipid peroxidant generation and enhance mitochondrial membrane resistance to oxidative stress and increases mitochondrial function.[39] Along with increased bilirubin, induction of HO-1 by CoPP also leads to increased production of CO. Recent studies have demonstrated that CO can directly reduce the activity of NADPH oxidase and lower O_2^- production in vascular smooth muscle cells reviewed by Abraham and Kappas.[2] CO and biliverdin protect vascular function and endothelial cell sloughing in rats with type I diabetes.[40] It is also possible that induction of HO-1 with CoPP can increase the levels of other antioxidant proteins such as catalase or superoxide dismutase to lower O_2^- production in the kidney of radiocontrast-induced AKI rats, as similar observations were recently made in the kidney of angiotensin II hypertensive mice, diabetic rats, or HO-2siRNA rats treated with CoPP.[41–43]

Compared to controls, heme levels were significantly increased in the kidneys of AKI rats. CoPP administration reduced the increase in heme to levels that were even below those observed in control rats. Conversely, the inhibition of HO activity with SnMP significantly increased heme levels compared to nontreated AKI rats. Induction of HO-1 has been previously shown to be protective to the kidney in models of AKI in which heme levels were increased.[6] Thus, the decrease in free heme content following CoPP may be another mechanism by which CoPP decreases oxidative stress and protects against radiocontrast-induced AKI. The physiology and pathology of HO-1/HO-2 in kidney disease has recently been reviewed by Tracz *et al.*[44]

Radiocontrast-induced AKI was associated with a marked increased in iNOS protein in the kidney. This was attenuated by CoPP treatment, which is in agreement with previous studies examining the effect of HO-1 induction on iNOS levels in diabetes.[31] Induction of iNOS is a classical marker of macrophage infiltration and increased inflammation in the kidney. Previous studies have reported that inhibition or knockout of the iNOS gene prevents the development of renal injury after ischemia/reperfusion injury.[45,46] High levels of iNOS can also increase the levels of nitrosative stress through the generation of peroxynitrite. Peroxynitrite can cause protein nitrotyrosine modification, which can weaken cellular antioxidant defenses, furthering renal injury. Thus, reduction of nitrosative damage in radiocontrast-induced AKI via the lowering of iNOS levels may play a role in the protection afforded by induction of HO-1 with CoPP.

Another key observation is that upregulation of HO-1 by CoPP decreased activation of caspase and pro-apoptotic Bax protein levels as well as increased the levels of Bcl-xl and Bcl-2 proteins, which are important for cell survival. The changes in caspase activity, Bax, Bcl-2, and Bcl-xl appear to be dependent on the total HO activity (HO-2 and HO-1). SnMP inhibits HO activity, which led to a marked increase in BAX and inhibits BCL-XL. Since SnMP caused substantial inhibition of Bax and caspase activity, this suggests that HO-2 plays a major role in regulating pro-apoptosis pathway since SnMP caused dramatic increases in Bax levels. HO-2 deficiency has been shown to contribute to an increase in O_2^- anion and renal dysfunction in HO-2 knockout mice.[47] Silencing HO-2 has been shown to increase caspase and decreases extracellular superoxide dismutase.[41,42] Recently, Kim *et al.*[8] have shown that induction of HO-1 protects the rat kidney from ureteral obstruction via an antiapoptotic pathway. The anti-apoptotic Bcl-2 and Bcl-xl proteins have been shown to regulate mitochondrial cytochrome *c* release and caspase activation during oxidant injury.[48,49] Enhancement of these specific survival signals may lead to the preservation of renal function during oxidant injury, as was observed in this study of radiocontrast-induced AKI. Previous studies have shown that the anti-apoptotic effects of CO depend upon both the phosphatidylinositol 3-kinase/Akt and the p38 mitogen-activated protein kinase signaling pathways, which are able to enhance signal transducers and activators of transcription 3 activation and attenuate caspase activity.[19,50]

The kidney medulla has been shown to be particularly vulnerable to radiocontrast injury.[28,51] Interestingly, our model revealed a difference in AKI-elicited HO-1 induction between the medulla and cortex. HO-1 was induced in the cortical but not in the medullary regions of radiocontrast-

treated kidneys. Importantly, the lack of HO-1 in the medulla was associated with activation of caspases and increased Bax levels; both effects were absent in the cortex. These results suggest that cortical induction of HO-1 may represent a local, endogenous, anti-apoptotic mechanism in radiocontrast-induced AKI, but the medullary response requires a strong inducer, such as CoPP, to raise medullary HO-1 to protective levels. Considering that the S3 segment of the proximal tubule and the thick ascending limb of the loop of Henle (TALH) are located in the medulla, it is not surprising that these segments are involved. Previous work of ours[52] has shown that TALH cell survival after experiencing oxidative stress injury (via angiotensin II) may be facilitated by selective upregulation of HO-1, thereby, blocking inflammation and apoptosis. Chung and Perrella[53] emphasized in an editorial comment on that work that the role of HO-1 in renoprotection is very dependent on the location of the specific tubular cells that are responsive to HO-1.

In summary, our findings indicate that HO-1 prevents AKI-induced renal dysfunction by a mechanism involving a decrease in cellular heme as well as increases in CO and bilirubin. The increase in heme metabolism was associated with an increase in eNOS, and a decrease in iNOS. Thus, induction of HO-1 enables the kidney to mount an important host-defense response to resist the AKI-mediated increase in oxidative stress and the resultant renal dysfunction. The upregulation of HO-1 expression caused a decrease in the pro-apoptotic caspases and Bax levels, and increases in both Bcl-2 and Bcl-2 signaling proteins; which suggests that induction of HO-1-derived CO or bilirubin activates the pro-survival and inhibits the pro-apoptotic signaling pathways. These results suggest that HO-1 may be potentially beneficial strategy to prevent AKI in clinical circumstances such as the use of dye contrast in selected patients who might have a propensity toward sustaining such renal injury.

MATERIALS AND METHODS

Animals

Male Sabra rats (Wistar-derived colony), weighing 260–380 g, were used in all experiments. They were divided into four groups, six rats per group: (A) controls, (B) uninephrectomized, (C) uninephrectomized and pretreated with CoPP 1 mg/100 g body wt, subcutaneously, once 4 days before terminating the experiments, and (D) uninephrectomized and pretreated with SnMP, 5 mg/100 g body wt, subcutaneously, daily for 4 days before terminating the experiments. On day 0, under anesthesia with pentobarbital (50 mg/kg/body wt, intraperitonealy), the femoral artery was cannulated using a polyethylene catheter (PE 50; Clay-Adams, Parsippany, NJ, USA) that exited through the skin in the back of the neck to allow for blood sampling and injection of the contrast agent. Rats were housed in Nalgene metabolic cages (Nalge Co., Rochester, NY, USA), fed standard rat chow, and allowed free access to water except for the day of radiocontrast administration.

Development of AKI

To increase the likelihood of AKI, rats were subjected to reduction of renal mass by uninephrectomy 3 weeks before exposure to the contrast agent. All rats were then salt depleted by feeding a low sodium diet for 7 days before exposure to the contrast media while receiving daily treatment of furosemide (2 mg/kg) for 7 days. Prostaglandin production was also inhibited 1 h before contrast administration by a single intravenous injection of indomethacin (10 mg/kg). Sodium iothalamate contrast media (60% Angio-Conray, Mallinckrodt Inc., St Louis, MO, USA) was injected through the arterial cannula over approximately 2 min at the dosage of 8.0 ml/kg body wt. This dose of sodium iothalamate has previously been demonstrated to cause AKI in rats.[28]

Measurement of renal function and tissue harvesting

Renal function parameters were measured following administration of the contrast agent before killing. Under light halothane anesthesia, tail blood samples (0.5 ml) were collected into heparinized tubes and plasma was obtained following centrifugation. Urine was collected 24 h before sacrifice. Serum and urine creatinine were determined using standard assays (Sigma Creatinine kit 555A, Sigma, St Louis, MO, USA). Urine volume was also determined. At the time of killing, animals were deeply anesthetized with ketamine/xylazine/acepromazine (2.0/0.6/0.3 mg/kg). Kidneys were removed and cut bilaterally; one-half was snap frozen in liquid nitrogen and stored at -70°C for subsequent biochemical analysis.

Cortex and outer medulla preparation

Kidneys were perfused with ice-cold hydroxyethylpiperazine-*N*′-2-ethanesulfonic solution, sliced, and dissected to separate the cortex and outer medulla as described previously.[54] Tissue segments were homogenized (4 ml/g wet weight) in 10 mM Tris buffer (pH 7.5) containing 0.25 M sucrose. The homogenates were centrifuged at 27 000 *g* for 20 min at 4°C. The supernatant was centrifuged at 105 000 *g* for 1 h at 4°C, and the resulting microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.6).

Western blotting

For tissue homogenates, 30 μg of protein was combined with sample buffer and separated on an sodium dodecyl sulfate-polyacrylamide gel. Gel percentages were 8% for proteins with molecular weights of 60 kDa and above, 12% for proteins with molecular weights of 25–60 kDa, and 15% for molecular weights less than 25 kDa. For immunoblotting, the separated proteins were electrophoretically transferred to PVDF membranes for 70 min at a constant 50 mA per membrane. The membranes were then blocked with 10% milk in 10 mM Tris base, 150 mM NaCl, and 0.1% Tween at 4°C overnight. After being washed with Tris-buffered saline with Tween, the membranes were incubated with 1:1000 dilutions in 3% milk in Tris-buffered saline with Tween or commercial bovine serum albumin as per manufacturer's instructions for anti-HO-1 and anti-HO-2 (Stressgen Biotechnologies Corp., Victoria, BC, Canada), anti-eNOS and anti-iNOS (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-β actin (Sigma), anti-Bax, anti-Bcl-xl, and anti-Bcl-2 (Cell Signaling Technology Inc., Danvers, MA, USA) for 2 h at room temperature with constant shaking. The filters were rinsed twice, washed three times with Tris-buffered saline with Tween for 10 min, and then probed with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (anti-mouse for β-actin) at dilutions of 1:2000 for 1 h at room temperature with constant shaking. The filters were again rinsed twice and washed three times with Tris-buffered saline with Tween for 10 min. Chemiluminescence detection was performed with the Amersham ECL Plus Detection kit (Amersham, Piscataway, NJ, USA) according to the manufacturer's instructions.

Measurement of HO activity and heme content

Tissues were homogenized (4 ml/g wet weight) in homogenization buffer (pH 7.4), containing 0.25 M sucrose. The homogenates were centrifuged at 10 000 *g* for 10 min at 4°C. The supernatant, containing cytosolic biliverdin reductase, was then used for measuring HO activity. HO activity was assayed by a method in which bilirubin, the end product of heme degradation, is extracted with chloroform and its concentration is determined spectrophotometrically (Dual UV/VIS Beam Spectrophotometer λ 25; Perkin-Elmer, Norwalk, CT, USA) using the difference in absorbance at wavelength from λ 460 to λ 530 nm with an absorption coefficient of 40 mM^{-1} and cm^{-1}.[55] Heme content was determined by the pyridine hemochromogen method as described previously.[55] The absorbance difference between λ 557 and λ 530 nm was used to calculate the heme content using an extinction coefficient of 20.7 mM^{-1} and cm^{-1}.

O_2^- anion production

O_2^- production was assayed by the spectrophotometric measurement of Ferricytochrome *c* reduction. Kidney homogenates were frozen (−80°C) until use. Tissue homogenates were incubated with 0.5 ml of reaction mixture, consisting of Krebs Ringer phosphate buffer containing 80 μM cytochrome *c* and 2 mM NaN_3. After 1 h of incubation at 37°C, the supernatants were collected and used to assay the amount of reduced cytochrome *c* by the difference in absorbance at 550–468 nm. O_2^- release was calculated using a coefficient of 0.0245 (the extinction coefficient μM/l of cytochrome *c* determined at 550–468 nm), and expressed as μmol O_2^-/mg protein.

Caspase activity assay

Caspase-3 and caspase-9 activity was determined using colorimetric assays (ApoTarget kit) following the manufacturer's protocol (BioSource International, Camarillo, CA, USA). Briefly, tissue cell lysates were prepared in cell lysis buffer (Tris-buffered saline containing detergent) and protein concentrations in samples were estimated using the Bradford method. Then, 200–300 μg of protein lysate per sample was mixed with 200 μM substrate in 2 × reaction buffer (DEVD-*p*NA for caspase-3 and LEHD-*p*NA for caspase-9) and incubated at 37°C overnight in the dark. Developed color was measured at 405 nm in a microplate reader (Bio-Rad, Hercules, CA, USA). Blank readings were subtracted from each sample before calculation. Caspase activity was expressed in terms of absorbance units (OD 405 nm) per milligram of protein.

Statistical analysis

The data are presented as mean ± s.e. for the number of experiments. Statistical significance ($P < 0.05$) between the experimental groups was determined by the Fisher methods for multiple comparisons. For comparison between treatment groups, the null hypothesis was tested by single factor analysis of variance for multiple groups, or unpaired *t*-test for two groups.

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